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TITLE: VIPER: Chronic Pain after Amputation: Inflammatory Mechanisms, Novel Analgesic Pathways, and Improved Patient Safety

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14. ABSTRACT
Chronic pain is a significant problem after nerve injury from trauma or surgery. Current therapies and attempts at prevention have proven largely ineffective. Through analysis of data obtained in the Molecular Signatures of Chronic Pain Subtypes study termed Veterans Integrated Pain Evaluation Research (VIPER) (W81XWH-11-2-0003) we have discovered two novel pain pathways with potential therapeutic relevance (Wnt and TGR5). In addition, we recognize that improving the safety and efficacy of existing therapies must continue to be a priority and plan to use the large pharmacogenomic database at Vanderbilt University to identify patients at risk for adverse opioid related events. The current proposal intends to study the contribution of non-neuronal immune cells (macrophages) to chronic pain while also evaluating novel analgesics in relevant animal models. The current proposal also attempts to determine the optimal patient population for opioid therapy while identifying those patients at greatest risk from opioids.

15. SUBJECT TERMS
Post-amputation pain, Phantom limb pain, Residual limb pain, neuropathic pain, novel analgesics, opioid related adverse events.

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INTRODUCTION:
Chronic pain is a significant problem after nerve injury from trauma or surgery. Current therapies and attempts at prevention have proven largely ineffective. Through analysis of data obtained in the Molecular Signatures of Chronic Pain Subtypes study termed Veterans Integrated Pain Evaluation Research (VIPER) (W81XWH-11-2-0003) we have discovered two novel pain pathways with potential therapeutic relevance (Wnt and TGR5). In addition, we recognize that improving the safety and efficacy of existing therapies must continue to be a priority and plan to use the large pharmacogenomic database at Vanderbilt University to identify patients at risk for adverse opioid related events. The current proposal intends to study the contribution of non-neuronal immune cells (macrophages) to chronic pain while also evaluating novel analgesics in relevant animal models. The current proposal also attempts to determine the optimal patient population for opioid therapy while identifying those patients at greatest risk from opioids.

KEYWORDS:
Post-amputation pain, Phantom limb pain, Residual limb pain, neuropathic pain, novel analgesics, opioid related adverse events.

ACCOMPLISHMENTS:
What were the major goals of the project?

Goal 1: Characterize the role of Wnt signaling in macrophage polarization, mouse nerve injury models and human neuroinflammation.

Major Task 1: Characterize macrophage polarization changes after Wnt signaling modification in mouse macrophage cell culture – **80% complete**

Major Task 2: Determine the specific wnt pathway responsible for prevention of mechanical allodynia in a mouse model of peripheral nerve injury and correlate this with macrophage polarization state and IL-6 to IL-10 ratio – **50% complete**

Major Task 3: Characterize wnt pathway expression and DNA methylation changes in humans before and after amputation and determine the role of cytokine ratio measurement in prediction of pain phenotype - **75% complete**

Goal 2: Determine the role of TGR5 in astrocyte activation and treatment of mechanical allodynia in a mouse model of neuropathic pain.

Major Task 1: Determine role of TGR5 signaling in astrocyte activation – **100% complete**

Major Task 2: Determine the role of TGR5 signaling in treating mechanical allodynia in a mouse peripheral nerve injury model - **80% complete**

Goal 3: Use existing data from the Vanderbilt EMR and genotyping repositories to look for associations between genetic variants and pain phenotypes

Major Task 1: Preliminary analyses conducted to confirm the precise numbers of patients for whom there are sufficient data available. Validation of previously published genotype-phenotype associations – **75% complete**

Major Task 2: Discovery and validation of novel exomic variants associated with opioid adverse drug events – **0% complete**

What was accomplished under these goals?

Overall Progress

We are progressing as expected with the organization, experiments and logistics of this project. Following is a detailed list outlining accomplishments for this quarter.

- We have found that TGR5 agonist given intraperitoneally reduced mechanical allodynia on day 7 after injury.

- UHSHS has completed the first stage of qPCR analysis on VIPER blood samples showing significant differences in one wnt pathway component between cases and controls and close to significant differences in two other components. We are now following this up by running wnt pathway qPCR arrays on VIPER valproate patient samples before and after surgery and with and without pain. (Table 1)
    - We have spent the last three months extracting RNA from over 200 VIPER valproate samples.
    - In order to perform wnt array analysis, we have to amplify the RNA using a NuGen Trio amplification kit. This kit has been purchased by UHSHS.
    - They will begin by running 96 RNA samples (48 patients at two timepoints)

Table 1: Expression analysis of selected wnt pathway constituents in patients with and without residual limb pain enrolled in the Veterans Integrated Pain Evaluation Research (VIPER) study.

wnt pathway gene	Case	Control	p-value
APC	0.928	1.018	0.11
CTNNB1	0.9	1.018	0.05
FZD1	0.884	1.024	0.06
FZD3	1.183	1.081	0.25
LRP-3	1.152	1.118	0.4
LRP6-3	1.579	1.267	0.08

- Aim 3 of this grant focuses on identifying perioperative patients in the Synthetic Derivative Database at Vanderbilt University Medical Center who have experienced an adverse event related to opioid drug administration, and then accessing their genetic data from the BioVU resource to discover variants that are associated with opioid toxicity.

  Clarity of phenotype is of critical importance in genetic association studies, and as such we have settled on administration of naloxone as an objective way of identifying those patients with a high likelihood of having experienced such an event (respiratory depression). A secondary phenotype (constipation) identified by administration of gastric motility enhancing drugs is also being used to facilitate a sensitivity analysis of this definition.

  The group has met monthly either in person or by phone, and our progress is summarized below.

Regulatory

We have received approval from the VUMC IRB to extract the data from the various repositories, to merge the data and curate the dataset for analysis; and to conduct the genetic association analysis that will permit identification of variants associated with opioid toxicity. **Threats to completion: NONE**

Data assets
The dataset for analysis will include the following patient characteristics:

- Age
- Gender
- Race
- All CPT codes
- All ICD codes and comorbidities
- All preoperative medication dosing
- Perioperative Morphine Milligram Equivalents
- Motility medication dosing
- Naloxone dosing (and presence/absence of rapid response team code)

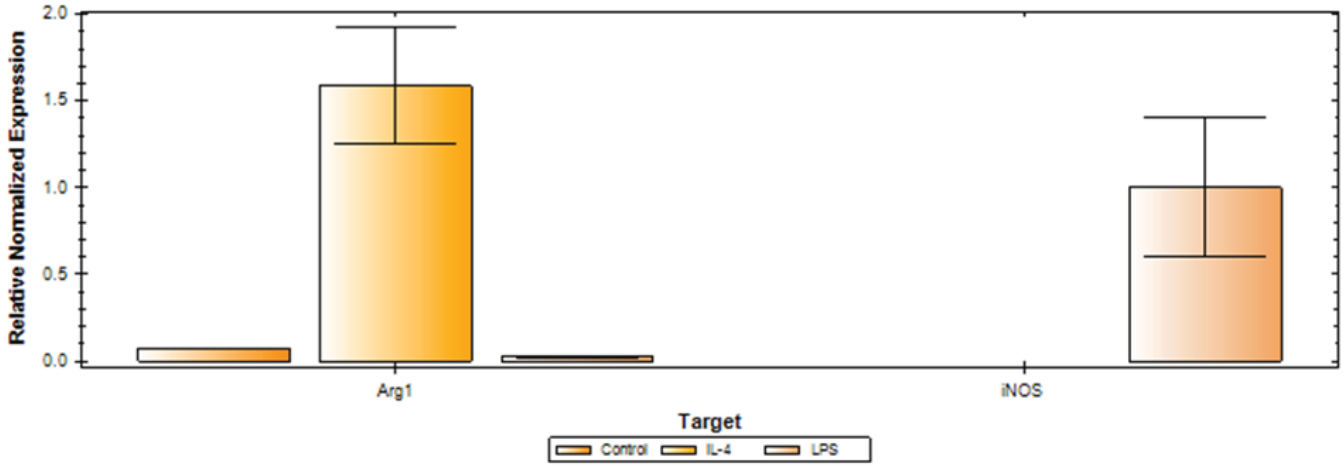
From our data sources, we have identified **3,654** individuals who have had:
1) A perioperative CPT code
2) A naloxone dose associated with a perioperative CPT code (within 2 days prior to and five days after)
3) Another **149,000** have had a motility drug dosed in that same interval.
4) **1.2M** people have had a perioperative CPT code ever – we would draw a control comparison from this cohort.

We are thus in good shape from the perspective of both clarity of phenotype and statistical power.
Threats to completion: NONE

Genetic assets and association study plans
We (ie Vanderbilt) have been running MEGAex assays on our stored BioVU samples on an ongoing basis, and to date (Sept 2017) we have these genetic data (covering over 2,000,000 genetic markers) on over 40,000 patients. The perioperative data we have obtained will be merged with these genetic data, and this pool of patients will provide us with adequate power to test hypotheses regarding genetic predictors of opioid-related adverse events (i.e., respiratory depression) at a near GWAS level. **Threats to completion: NONE**

- Macrophage sorting by phenotype using flow cytometry has not been as straightforward as hoped. We have tested another method (antibody bound magnetic beads) to separate macrophages by phenotype and have achieved success. This is not a major change in the direction of the science, just a change in the method of isolating macrophages (Please let me know if it requires an amendment to our research protocol or SOW). The magnetic bead sorting was done on macrophages collected from mouse peritoneum and treated with a known classic (M1) macrophage stimulating compound (LPS) and an alternative (M2) stimulating compound (IL-4). Macrophages were then sorted by beads containing antibodies to CD14 (a macrophage marker) and then qPCR was performed to confirm that stimulation produced the phenotype of interest. For PCR we targeted the M1 phenotype marker ARG1 and the M2 phenotype marker iNOS. Figure 1 below shows that we successfully created M1 and M2 macrophages, were able to isolate those macrophages and perform PCR on RNA from those macrophages that can distinguish M1 and M2 phenotype.
    - We will now repeat this experiment using wnt agonists as the stimulating agents.

Figure 1:



- Magnetic bead sorting of macrophages stimulated by compounds known to produce M1 and M2 phenotype was competed during the year. We have made a large amount of progress this quarter completing this task. The chart below shows a summary of all data collected. Results suggest that canonical wnt pathway ligands (wnt3a and gsk3 inhibition) mildly favor the M1 phenotype in mouse peritoneal macrophage culture. Interestingly the non-canonical wnt pathway ligand (wnt6) strongly favors M2 phenotype in these cells. If this result is confirmed in the pain model in Major Task 2 below we will have strong evidence that manipulating the non-canonical wnt pathway can prevent chronic neuropathic pain.

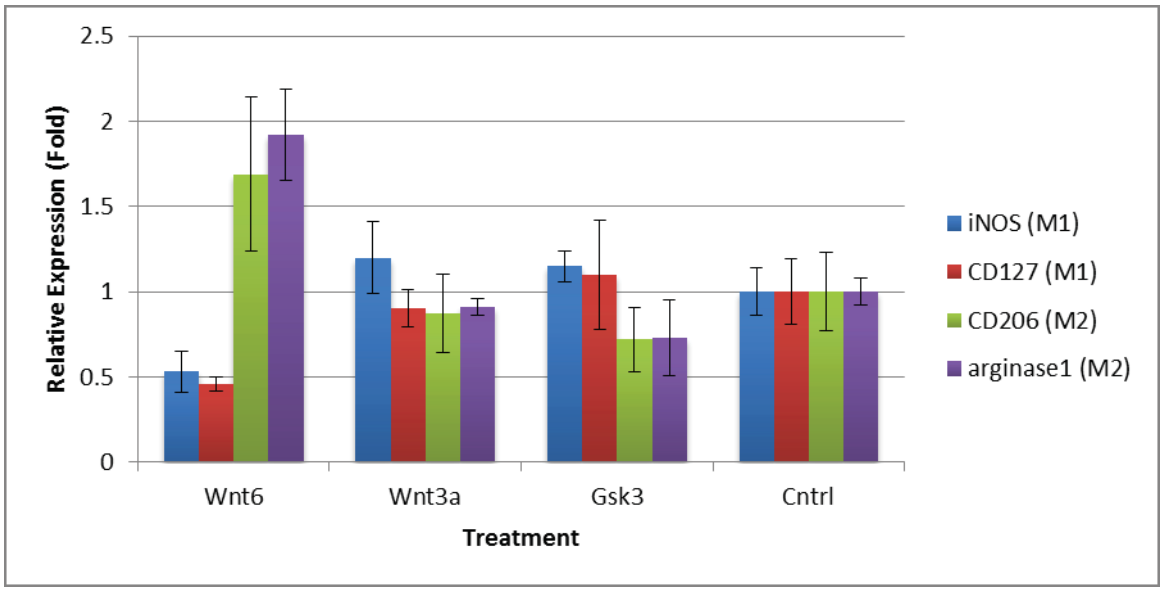
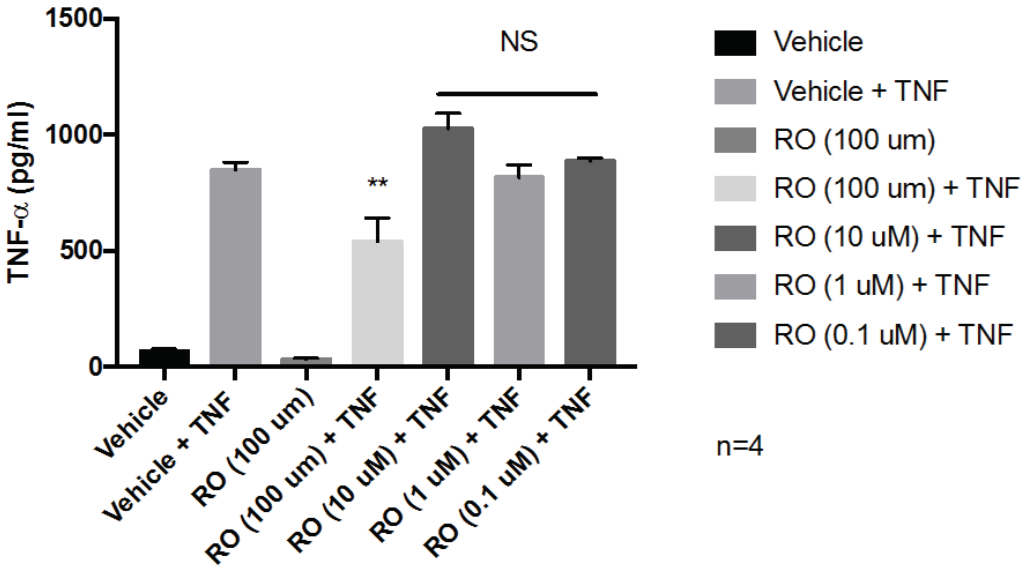


Figure 2.) Non-canonical wnt ligand Wnt6 favors M2 macrophage phenotype. Murine peritoneal macrophages were collected and cultured and treated with either wnt pathway ligands (wnt6 100ng/ml, wnt3a 100ng/ml, gsk3 inhibitor 100ng/ml or saline as control). RNA was collected and qPCR performed. Transcript levels were first normalized to GAPDH as a reference gene, and then to control for comparison to various treatments. All data are mean ± SEM (n=3, treatments and control).

- Astrocyte culture work has been completed showing a significant decrease in astrocyte activation at the highest dose of the TGR5 agonist (Figure 1 below). This is a very interesting result that strengthens our argument that the longer term, more chronic portion of the transition from acute to chronic pain is modulated by the TGR5 pathway through spinal cord astrocytes. We will, however, repeat this experiment to ensure reproducibility for a total “n” of 8.

Figure 3:



- Supernatant from the macrophage cultures used in Major Task 1 above were subjected to ELISA for IL-6 and IL-10. The results are shown in Figure 2 below. Results support the findings from Major Task 1 above. The noncanonical wnt ligand (wnt6) shown above to favor M2 macrophage phenotype also leads to increased IL-10 and decreased IL-6 in the culture supernatant. There is no significant change with either wnt3a or the gsk3 inhibitor.

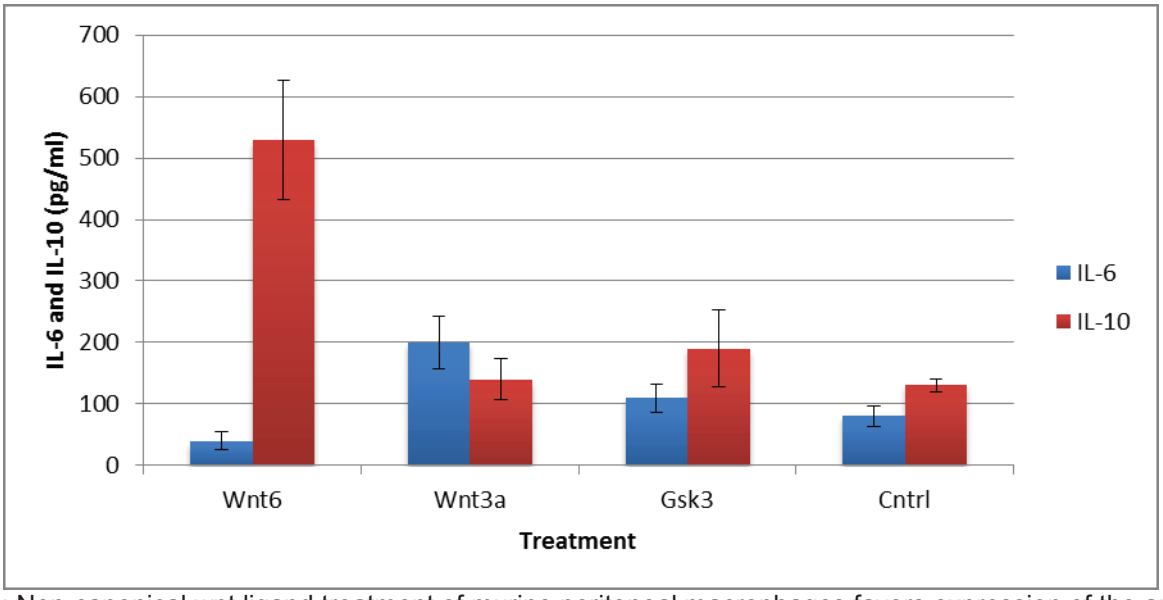


Figure 4: Non-canonical wnt ligand treatment of murine peritoneal macrophages favors expression of the anti-inflammatory cytokine IL-10. Supernatant from murine peritoneal macrophage cultures were collected after treatment with either wnt pathway ligands (wnt6 100ng/ml, wnt3a 100ng/ml, gsk3 inhibitor 100ng/ml or saline as control) or control and ELISA for IL-6 and IL-10 performed. All data are mean ± SEM (n=3, treatments and control).

What opportunities for training and professional development has the project provided?
Nothing to Report

How were the results disseminated to communities of interest?
Bile Acid Signaling and Neuropathic Pain. Can Agonism of the G-Protein Coupled TGR5 Receptor Modulate the Development of Chronic Pain?
Hendrickson, Kieber, Hsia, Buchheit, McDuffie, Buckenmaier, Shaw, Ji, Van de Ven
Duke Department of Anesthesiology Academic Evening 5/7/2016

Chamessian A, Qadri Y, Cummins M, Berta T, Hendrickson M, Buchheit T, Van de Ven T, "5-hydroxymethylcytosine (5hmC) and Ten-eleven translocation 1-3 (TET1-3) proteins in the dorsal root ganglia: expression and dynamic regulation in neuropathic pain." Somatosens Mot Res. 2017 Jun;34 (2):72-79

What do you plan to do during the next reporting period to accomplish the goals?
Description of work to be performed/completed during the next reporting period
During the next three months, we expect:

- Complete wnt PCR arrays on 48 patients at 2 timepoints (96 total samples)
- Be close to completion of the validation phase of Specific Aim 3 (genotype-phenotype associations to opioid related adverse events.)
- Start behavioral testing on mice treated with wnt pathway ligands in a spared nerve injury model with aim to complete this task by Year 3 quarter 2.

IMPACT:
What was the impact on the development of the principal discipline(s) of the project?
Pain medicine is limited by the limited number of new analgesics and adverse effects of opioids. Over the past year we have confirmed that TGR5 is important in both inflammatory pain and neuropathic pain and that a TGR5 agonist reduces the sensitivity in an animal pain model. There is a long road before therapies like this can be used in humans but we are taking the first steps.

Also, we were surprised at the proportion of patients with opioid related adverse events found using our search algorithm in the Vanderbilt database. Identifying this cohort preoperatively will allow not prevention of adverse events in the hospital and perhaps reduction of post-discharge opioid use which has been shown to lead to long-term opioid use in a subset of patients.

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
We are still early in this project and results have not been published although there is preliminary evidence that TGR5 agonists are able to treat mechanical allodynia in mice.

CHANGES/PROBLEMS:
Nothing to report

Changes in approach and reasons for change
There were no significant changes in approach. Minor changes include additional validation of flow cytometry results using qPCR of known targets that distinguish M1 from M2 phenotype in macrophages.

Collection of macrophages for PCR was done using antibody bound beads instead of flow cytometry and this worked very well.

Actual or anticipated problems or delays and actions or plans to resolve them
No delays or anticipated problems

Changes that had a significant impact on expenditures
No significant changes on expenditures

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
No changes or deviations

PRODUCTS:
Published
Accepted:

1. Chamessian A, Qadri Y, Cummins M, Berta T, Hendrickson M, Buchheit T, Van de Ven T, "5-hydroxymethylcytosine (5hmC) and Ten-eleven translocation 1-3 (TET1-3) proteins in the dorsal root ganglia: expression and dynamic regulation in neuropathic pain." Somatosensory & Motor Research

Published:

2. Alexander Chamessian[5], Thomas Van de Ven*[1,6], Thomas Buchheit[1,6], Hung-Lun Hsia[1,6], Mary McDuffie[7], Eric Gamazon[3], Colin Walsh[4,8], Stephen Bruehl[2], Chester 'Trip' Buckenmaier III[7,9], Andrew Shaw. Differential Expression of Systemic Inflammatory Mediators in Amputees with Chronic Residual Limb Pain. January 2017 - Volume 158 - Issue 1 - p 68–74

Books or other non-periodical, one-time publications.
Nothing to report

Other publications, conference papers, and presentations.
Nothing to Report

Website(s) or other Internet site(s)
Nothing to report

Technologies or techniques
Nothing to report

Other Products
Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?
Name: Thomas Van de Ven
Project Role: Principal Investigator
Nearest person month worked: 4.58
Contribution to Project: Coordinates all aspects of the project and assumes overall responsibility for its success.

Name: Ru-Rong Ji
Project Role: Co Investigator
Nearest person month worked: 0.48
Contribution to Project: He is responsible for interpreting and troubleshooting the proposed animal behavioral testing and cell culture experiments and his lab provides deep expertise in all experimental procedures

Name: Alexander Chamessian
Project Role: Graduate Student
Nearest person month worked: 12
Contribution to Project: He is responsible, along with Dr. Van de Ven, for completion of all animal behavior and cell culture experiments.

Name: Rachel Morales
Project Role: Program Manager
Nearest person month worked: 1.80
Contribution to Project: Overall project manager for all aspects of the proposal, including coordination of the biological samples, shipment of samples between sites and data organization, and ensures that the supplies are ordered and available

Name: Thomas Buchheit
Project Role: Co Investigator
Nearest person month worked: 0.24
Contribution to Project: Works closely with Dr. Van de Ven on all aspects of the project
Funding Support: Other resources

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report

What other organizations were involved as partners?
Organization Name: Vanderbilt University Medical Center
Location of Organization: 1161 21st Avenue South, Nashville, TN 37232-2520
Partner's contribution to the project: Collaborated in the research

Organization Name: Henry M. Jackson Foundation for the Advancement of Military Medicine Inc.
Location of Organization: 6720 A Rockledge Drive, Bethesda, MD 20817
Partner's contribution to the project: Collaborated in the research

SPECIAL REPORTING REQUIREMENTS
QUAD CHARTS:
Attached

APPENDICES:
Attachment 1- Quad Chart

VIPER II: Chronic Pain After Amputation: Inflammatory Mechanisms, Novel Analgesic Pathways, and Improved Patient Safety.

PI: Van de Ven, Thomas **Org:** Duke University **Award Amount:** $1,500,000

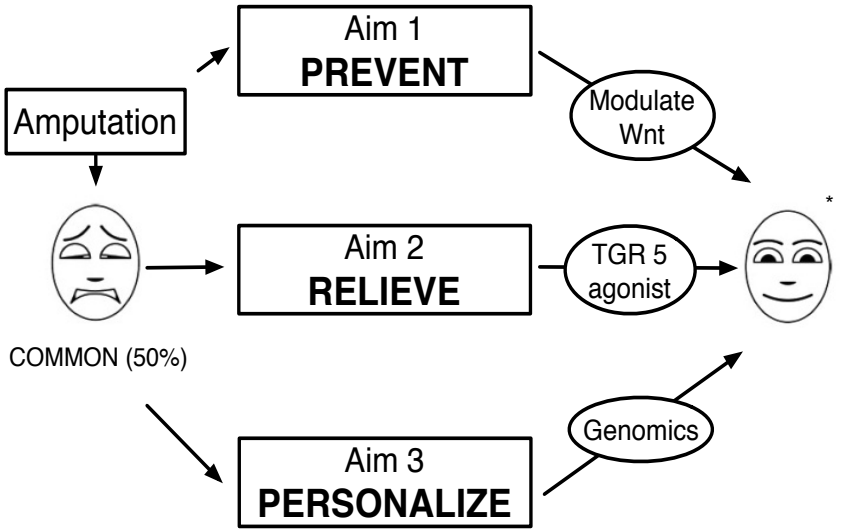
Study Aims

Problem: Current therapies for residual limb pain are ineffective or produce significant side effects.

Hypotheses: 1) Biomarkers found in the Veterans Integrated Pain Evaluation Research Study (VIPER) will lead to novel analgesics. 2) Pharmacogenomic profiling will improve the safety and effectiveness of current analgesics.

Approach

Convergent analysis of VIPER data show both the TGR5 and Wnt pathways to be important in chronic residual limb pain. We will:
1) Define the role of Wnt signaling in inflammation and mechanical allodynia after nerve injury using cell culture and animal models.
2) Test the effectiveness of TGR5 pathway agonists for the treatment of allodynia after nerve injury using animal models.
3) Use human pharmacogenomic predictors to improve the safety and effectiveness of current opioid treatments.



Timeline and Cost

Activities CY	15	16	17	18
Aim 1: Wnt - Cell culture, animal behavioral testing and cytokine measurement.				
Aim 2: TGR5 – Animal behavioral testing and cell culture experiments				
Aim 3: Pharmacogenomic analysis				
Reports (▮) and Manuscripts (♦)				
Estimated Total Budget ($K)	$200K	$500K	$500K	$300K

Goals/Milestones

CY16 Goals
- ✓ Begin macrophage polarization and astrocyte activation experiments
- ✓ Begin designing data capture for pharmacogenomic analyses
- ✓ Begin animal behavioral TGR5 experiments

CY17 Goals
- ✓ Begin wnt pathway human gene expression analysis
- ✓ Complete cell culture experiments
- ✓ Begin pharmacogenomic validation experiments
- ✓ Begin pharmacogenomic discovery experiments

CY18 Goals
- ✓ Complete ELISA and flow cytometry experiments
- ✓ Continue animal behavioral testing
- ❑ Continue pharmacogenomic discovery experiments
- ❑ Complete one manuscript

CY18 Goals
- ❑ Complete all experiments
- ❑ Complete two manuscripts
- ❑ Develop follow-on studies and apply for follow-on funding